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TITLE: Targeting L-Selectin to Improve Neurologic and Urologic Function After Spinal Cord Injury

PRINCIPAL INVESTIGATOR: Linda Noble

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, CA 94118-6215

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14. ABSTRACT

Purpose: We are evaluating the efficacy of diclofenac (DFA), an anti-inflammatory agent with L-Selectin sheddase activity, in a murine model of spinal cord injury.

Scope: These studies have focused on the efficacy of DFA in the context of dose, optimal therapeutic window, and dependency on injury severity, using clinically relevant outcome measures that include neurologic assessments and assays of bladder function.

Major findings:

- We demonstrated that 40 mg/kg DFA is the minimally effective dose to induce L-selectin shedding in a mouse model of spinal cord injury
- We demonstrated locomotor recovery in mice receiving 40mg/kg DFA up to 3 hours following spinal cord injury
- We demonstrated improved locomotor recovery using this paradigm for two injury severities, mild and severe, suggesting a robust therapeutic effect
- We identified no adverse effects to animal health, as evaluated by body weight
- We identified no added locomotor recovery due to multiple, successive doses of DFA. Moreover, additional doses proved to be toxic and increase animal mortality
- We have demonstrated improved white matter sparing in mice receiving 40 mg/kg DFA for up to 3 hours following spinal cord injury
- We have demonstrated reduced lesion volume in mice receiving 40 mg/kg DFA up to 3 hours following spinal cord injury.
- We have demonstrated no adverse effects of DFA on bladder function following spinal cord injury. However, the drug resulted in no improvement in bladder function

Significance: We have identified robust locomotor recovery in both mild and severe spinal cord injured mice that received DFA up to 3 hours following injury. Furthermore, we identified no adverse effects utilizing this dose. Therefore, these promising data suggest that 40mg/kg DFA, administered within 3 hours of spinal cord injury, as a single dose could be an effective therapeutic intervention for spinal cord injury.

15. SUBJECT TERMS

spinal cord injury, L-Selectin, diclofenac, mouse, urologic function, neurologic function

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INTRODUCTION

This proposal is investigating the hypothesis that the anti-inflammatory drug diclofenac (DFA), acting as an L-selectin sheddase, will improve neurologic outcome and ameliorate neurogenic bladder dysfunction resulting from spinal cord injury (SCI). L-selectin is expressed on the surface of all leukocytes. Preliminary data using the L-selectin knockout (KO) mouse confirmed the dependency of L-selectin on neurologic recovery and thus served as the basis for pharmacologic targeting of this molecule in a murine model of SCI. The specific aims of this proposal are to define the minimal effective dose of DFA, the optimal window of therapeutic intervention for DFA, whether DFA administration improves bladder function, and if the efficacy of DFA is dependent on proteolytic cleavage of L-selectin.

Please note that each task is indicated in bold.

BODY

Specific Aim 1

Task 1. Define the minimal effective dose of DFA

1a. – 1c. Reported in previous year's update. Main findings summarized below:

- **40mg/kg DFA is the minimally effective dose to induce L-selectin shedding from leukocytes following spinal cord injury.**
- **Mice with mild SCI receiving 40mg/kg DFA showed significant locomotor recovery relative to the vehicle controls.**
- **Urologic function is not being altered by DFA in this mild injury paradigm. However, the ability to detect DFA-dependent effects on urological function could be increased in a more severe SCI paradigm that has a wider range of recovery.**

1d. Conduct morphometric analyses (spared white matter, glial scarring, serotonergic fiber tracts) of the cords, prepared from animals in 1c (months 13-15).

Due to the challenges in assessing locomotor recovery with a mild SCI, we elected to analyze tissue from mice receiving a more severe SCI from Task 2c. Male C57BL/6 mice were subjected to a 3g weight dropped 5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg; n=14) or vehicle (n=7), was administered 3 hours following SCI using a randomized, blinded design. At 6-weeks post-injury spinal cords were harvested and sectioned for histological analysis. Cross-sections from one cohort were eriochrome stained, imaged, and analyzed for white matter sparing and lesion volume (Figure 1). A Mann-Whitney test demonstrated that **mice with severe SCI that received 50 mg/kg DFA treatment at 3 hours after injury exhibited increased the total white matter volume ($p<0.05$) with reduced total lesion volume compared to vehicle-treated animals ($p<0.05$). At the injury epicenter, white matter volume was significantly greater in DFA-treated animals compared to animals receiving a vehicle injection ($p<0.01$). No significant difference was observed in the lesion volume at the epicenter ($p=0.1903$).**

Spinal cords from a second cohort were sectioned in the longitudinal plane, stained for serotonergic fiber tracts (5-HT), and are currently being analyzed. We estimate that this task will be completed in 1-2 months.

Specific Aim 2

Task 2. Determine the optimal window of therapeutic intervention for DFA.

2a - 2c. Reported in previous year's update. Main findings summarized below:

- **Administration of 40mg/kg DFA immediately after a severe SCI supports locomotor recovery. DFA administration did not adversely affect animal weight and overall health in a severe SCI paradigm. Furthermore, bladder thickness and weight did not appear to be influenced by DFA.**
- **Administration of 40mg/kg DFA 3 hours after a severe SCI supports locomotor recovery.**
- **Administration of 40mg/kg DFA 8 hours after a severe SCI does not support locomotor recovery. Therefore, the optimal therapeutic window for efficacy is within a time frame of less than 8 hours post injury.**
- **Administration of multiple doses of 40mg/kg DFA after a severe SCI do not confer any added benefits over a single dose of 40mg/kg DFA. Moreover, multiple doses increase the risk of toxicity and adverse side-effects, indicating a single dose regimen of 40mg/kg DFA is most appropriate clinically.**

Specific Aim 3

Task 3. Determine if DFA improves bladder function

3a. Using optimal dosing defined in 2c, compare urologic function in spinal cord injured mice treated with either vehicle or DFA. (months 27-29).

We have previously shown that long-term neurological recovery is improved in our optimal dosing regimen: a severe model of SCI with the minimally defined dose of DFA (40 mg/kg), administered 3 hours following injury (Task 2c). To determine if DFA treatment improves urologic function in the severe model of SCI, male C57BL/6 mice were subjected to a 3g weight dropped 5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg; n=15) or vehicle (n=13) was administered 3 hours following SCI using a randomized, blinded design. At 7-weeks post-injury awake cystometry was performed (Figure 2). An unpaired T-test demonstrated that **mice with severe SCI that received 50 mg/kg DFA treatment at 3 hours after injury exhibited no significant improvements compared to vehicle-treated control mice in several measures of bladder function including intermicturition interval (p=0.2), threshold pressure (p=0.4), minimal and maximum voiding pressure (p=0.3 and 0.6, respectively), residual urine (p=0.8), voiding efficiency (p=0.1), and the occurrence and pressure amplitude of non-voiding contractions (p=0.1 and 0.4 respectively).**

Taken together, these data demonstrate that mice with severe SCI receiving 40mg/kg DFA at 3 hours post-injury do not show significant improvement in urologic function compared to the vehicle controls.

Specific Aim 4

Task 4. Determine if efficacy of DFA is dependent on its proteolytic cleavage of L-selectin.

To assess the dependence of DFA-induced cleavage of L-selectin on the functional recovery observed in Tasks 1 and 2, we obtained L(E) same mice from a collaborator at another institution. The mice were successfully rederived and breeding pairs for set up. Endogenous shedding of L-selectin has been previously shown in response to painful stimuli and may play a role in inflammation following SCI. Therefore, an altered response to SCI may occur in L(E) same mice that are resistant to L-selectin shedding. Prior to assessing the efficacy of DFA in L(E) same mice, we compared neurological recovery following moderate-severe SCI in L(E) same versus WT mice. Male C57BL/6 (n=10) and L(E) same (n=7) mice were subjected to a 2g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level.

Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion weekly 6 weeks post-injury (Figure 3). A two-way repeated measures ANOVA demonstrated a significant effect for time

($p < 0.0001$), but no effect for genotype ($p > 0.05$). A Sidak's multiple comparisons test demonstrated **no significant difference between L(E) same mice versus wild-type mice with moderate-severe SCI at all time points ($p > 0.05$)**.

These data suggest that endogenous L-selectin shedding does not affect neurological recovery following moderate-severe SCI.

The next step is to confirm that DFA does not induce L-selectin shedding in L(E) same mice. Studies are currently underway to examine L-selectin shedding in response to administration of 40 mg/kg DFA in L(E) same mice. These studies will be complete in the next month. If not significant differences are observed between DFA and vehicle treated mice, long term neurological assessment of L(E) same mice treated with DFA or vehicle control will be performed. These studies should be complete in 3-4 months. If L-selectin shedding is observed, we will evaluate the effect of DFA on L-selectin shedding in the two parental transgenic mice lines (L(E) neo and L(E) homo) that are crossed to generate the L(E) same genotype.

Key Research Accomplishments

- **Identified 40mg/kg DFA as minimal effective dose required for L-selectin sheddase activity**
- **Demonstrated immediate administration of 40mg/kg following mild SCI results in improved locomotor recovery**
- **Demonstrated that 40mg/kg DFA does not have adverse effects on animal health**
- **Demonstrated efficacy of 40mg/kg DFA is retained following severe SCI**
- **Demonstrated optimal therapeutic window for 40/mg/kg DFA is up to at least 3 hours post-SCI (and possibly longer). However, no benefit is seen when treatment is initiated at 8 hours post – SCI.**
- **Demonstrated no benefit to multiple doses of DFA**
- **Demonstrated greater white matter sparing in severe SCI mice receiving 40 mg/kg at 3 hours post-injury**
- **Demonstrated reduced lesion volume in severe SCI mice receiving 40 mg/kg at 3 hours post-injury**
- **Demonstrated that DFA does not exacerbate bladder function following SCI**
- **Demonstrated similar neurological recovery in L(E) same mice versus wild-type mice**

Conclusions

- **40mg/kg DFA is the minimal effective dose to induce L-selectin shedding in the plasma and spinal cord following SCI**
- **40mg/kg DFA improves locomotor recovery in both mild and severe SCI when administered within 3 hours and has no adverse effects**
- **40mg/kg DFA improves white matter sparing following severe SCI when administered within 3 hours post-injury**
- **40mg/kg DFA reduces the lesion volume following severe SCI when administered within 3 hours post-injury**
- **DFA does not improve, nor exacerbate, bladder recovery following severe SCI**
- **Endogenous L-selectin shedding does not appear to be a critical factor in neurological recovery following SCI**

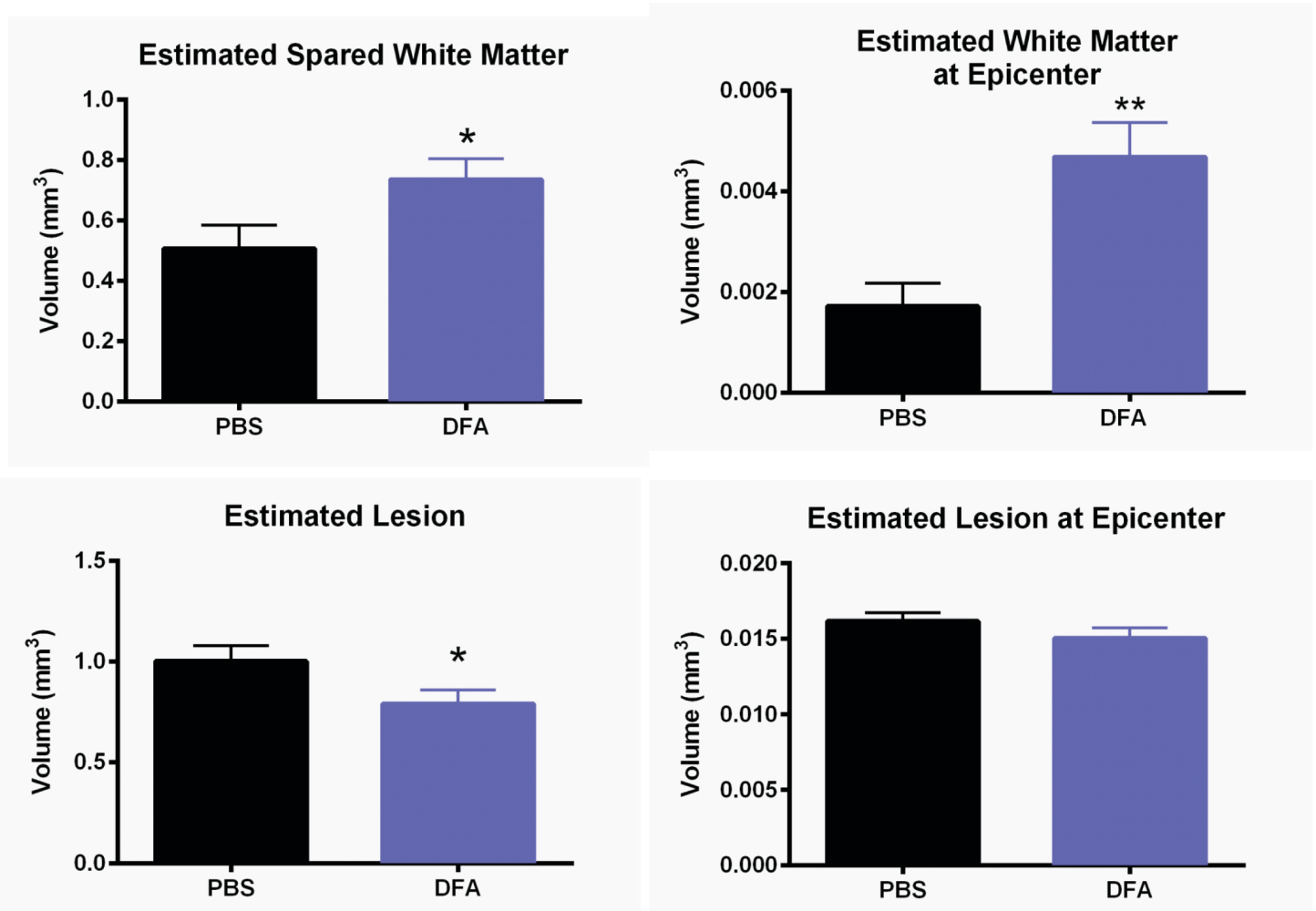


Figure 1: 40 mg/kg DFA administration 3 hours post-severe SCI enhances white matter sparing and reduces the lesion volume

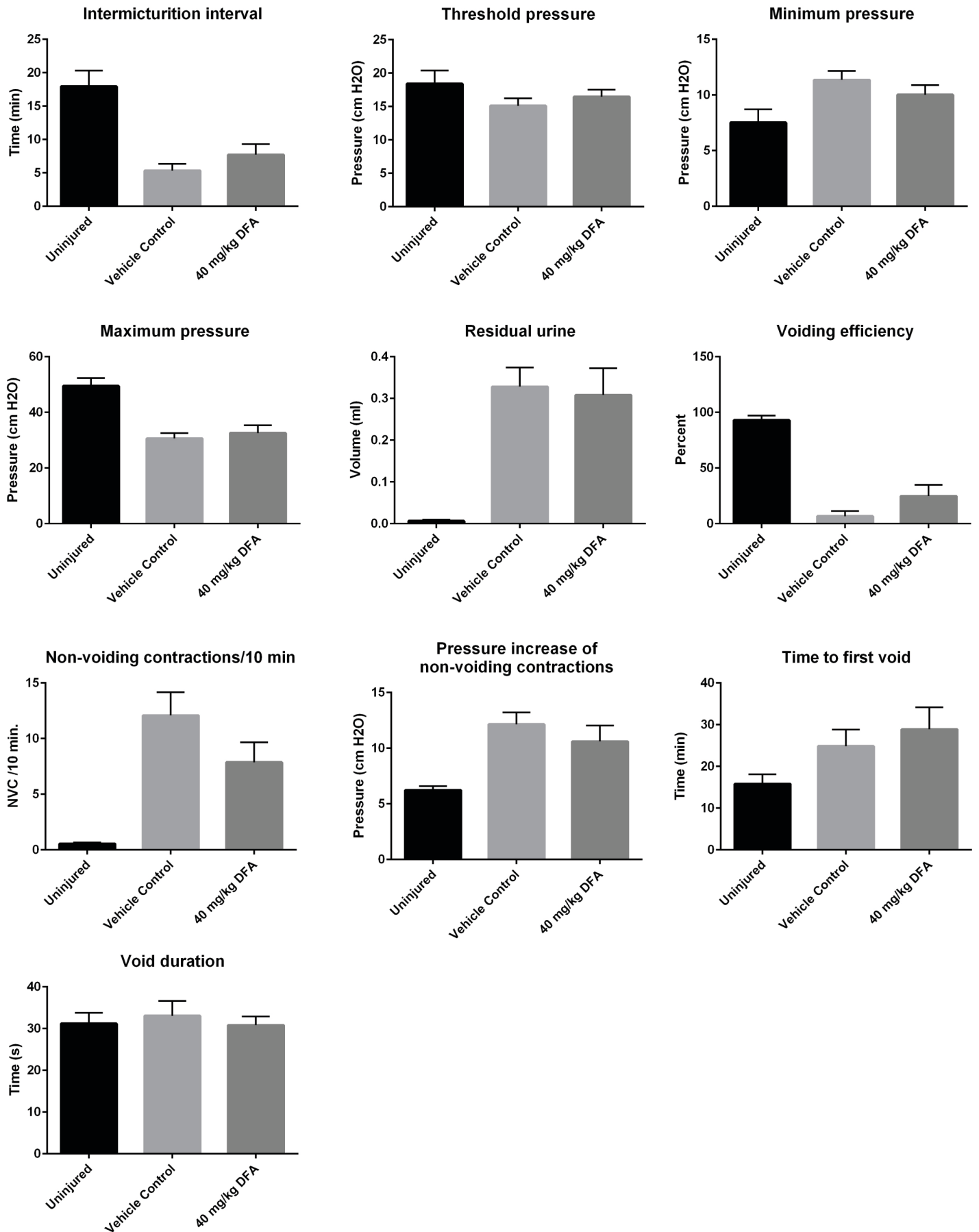


Figure 2: 40 mg/kg DFA administration 3 hours post-severe SCI does not alter urologic function compared to vehicle controls.

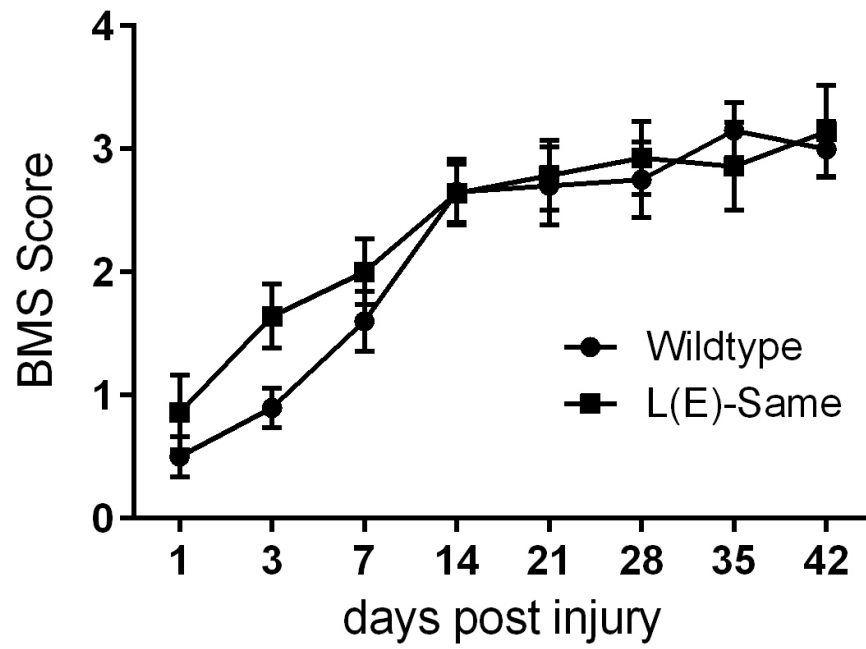


Figure 3: Similar neurological recovery in L(E) same mice versus wild-type mice following moderate-severe spinal cord injury

Targeting L-Selectin to Improve Neurologic and Urologic Function After Spinal Cord Injury

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Org: University of California San Francisco

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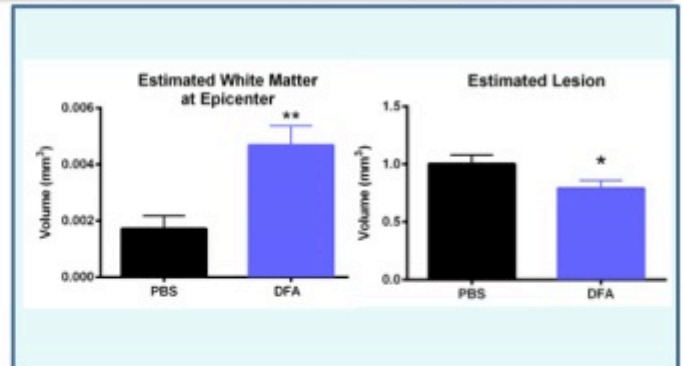


Study/Product Aim(s)

- Specific Aim 1: Define minimal effective dose of DFA
 - We determine that 40 mg/kg improves white matter sparing and reduces the lesion volume after severe spinal cord injury
- Specific Aim 3: Determine if DFA improves bladder function
 - We determine that DFA does not adversely exacerbate or improve bladder function after severe spinal cord injury
- Specific Aim 4: Determine if efficacy of DFA is dependent on its proteolytic cleavage of L-selectin.
 - We validated the transgenic line to be used for Aim 4

Approach

Awake cystometry was used to access bladder function in spinal cord injured mice that received either saline or 40mg/kg DFA at 3 hours after a severe model of spinal cord injury. White matter and lesion volume were measured from eriochrome stained sections from mice that received either saline or 40mg/kg DFA at 3 hours after a severe model of spinal cord injury.



Accomplishment: Administration of 40mg/kg DFA 3 hours post severe spinal cord injury significantly improves white matter sparing ($p < 0.01$) and significantly reduces the total spinal cord injury lesion volume ($p < 0.05$).

Timeline and Cost

Activities	CY	13	14	15	16
Define min. eff. DFA dose					
Test window/duration of DFA					
Evaluate urological recovery					
Evaluate L-selectin sheddase mechanism					
Estimated Budget (\$K)		\$251	\$260	\$272	\$000

Updated: 10/28/15

Goals/Milestones (Example)

CY13 Goal – Define Minimal Effective Dose

- ☒ Obtain IACUC approval
- ☒ Identify minimal effective DFA dose
- ☒ Demonstrate neurologic recovery with minimal DFA dose

CY14 Goals – Test Window/Duration of DFA

- ☒ Evaluate DFA 1/3/8 hours post-SCI
- ☒ Evaluate DFA 1, 2, and 3 days post-SCI
- ☒ Evaluate DFA in severe SCI

CY15 Goal – Production readiness

- ☒ Evaluate urological recovery post-DFA administration
- ☒ Investigate DFA induced L-selectin activity with transgenic mice

Comments/Challenges/Issues/Concerns

- Work for Specific Aim 4 is ongoing and will be completed during no-cost extension.

Budget Expenditure to Date

Projected Expenditure: \$782,320 (years 1 to 3 total budget)

Actual Expenditure: \$679,066.08 (Expenses through Sept 2015)